1986

Inhibition of Psychrotrophic Bacterial Growth in Refrigerated Milk by Addition of Carbon Dioxide

Robert Francis Roberts

INHIBITION OF PSYCHROTROPHIC BACTERIAL GROWTH IN REFRIGERATED MILK BY ADDITION OF CARBON DIOXIDE

By

ROBERT F. ROBERTS

A thesis submitted
in partial fulfillment of the requirements for the
degree of Master of Science
Major in Dairy Science
South Dakota State University
1986

INHIBITION OF PSYCHROTROPHIC BACTERIAL GROWTH IN REFRIGERATED MILK BY ADDITION OF CARBON DIOXIDE.

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

George S. Torrey — Date
Thesis Advisor

David J. Schingoethe — Date
Acting Head,
Dairy Science Dept.

DEDICATION

To my partner in all things, Elisabeth.

ACKNOWLEDGEMENTS

Appreciation is extended to Dr. G. Torrey for his input and guidance throughout the course of this research.

Dr. J. Parsons, Dr. R. Baer, Dr. K. Spurgeon, Prof. S. Seas, and the Production Faculty of Dairy Science are thanked for their contribution to my expeirence at SDSU.

The Department of Microbiology is thanked for use of various equipment and materials throughout my research. Dr. F. Seymor and Mr. F. Melorse are thanked for their help in rebuilding the Varian 1520 gas chromatograph. Dr. W. K. Gauger's advice on matters of taxonomy was appreciated. Thanks to Dr. W. L. Tucker for his help with statistical analysis.

My graditude to the Graduate Students in Dairy Science for their friendship and support.

Finally, the love and support of my wife, Elisabeth, my parents, my in-laws, my brothers and sisters, and all my relatives especially P. and C. Verret is acknowledged as the reason for my success in this endevor. Thanks to you all.

R. F. R.

TABLE OF CONTENTS

INTRODUCTION.....1

LITERATURE REVIEW.....3

Incidence of psychrotrophic bacteria in refrigerated raw milk.....3

Genera of psychrotrophs commonly isolated from raw milk.....6

Gram-negative bacteria.....6

Gram-positive bacteria.....6

Organisms of public health significance.....7

Defects produced by psychrotrophic bacteria in milk..8

Methods proposed to control growth of psychrotrophic bacteria.....9

Sanitation.....9

Refrigeration.....10

Thermization.....10

Preculturing milk with lactic acid bacteria....12

The lactoperoxidase/thiocyanate/peroxide system.....13

Addition of food additives.....15

Addition of carbon dioxide.....16

Use of carbon dioxide as a preservative in milk.....19

References.....24

ARTICLE: Inhibition of Psychrotrophic Bacterial Growth in Refrigerated Milk by Addition of Carbon Dioxide.....30

Abstract.....31

Introduction.....31

TABLE OF CONTENTS
(continued)

Materials and Methods.....33

Results and Discussion.....39

Acknowledgements.....45

References.....46

SUMMARY AND CONCLUSIONS.....59

APPENDIX A: Supplemental information.....62

APPENDIX B: Growth curves for isolates 1p, 2p, and 12p, and aerobic plate count data for Raw Milks 3 and 4, generated during the project and not included in the article.....65

LIST OF TABLES

Table — Page

Article

1 Characterization of psychrotrophic bacteria isolated from raw milk samples.....48

2 Carbon dioxide concentrations used in growth experiments in whole milk inoculated with pure cultures and incubated at 7°C.....49

3 Initial and final pH values for milks used in growth experiments with pure cultures incubated at 7°C.....50

4 Generation times (GT) and percent uninhibited growth rate for isolates incubated at 7°C in whole milk and whole milk treated with CO_2.....51

5 Carbon dioxide concentrations in growth flasks containing naturally contaminated raw milk.....52

6 Comparison of microbial populations in raw milks with and without added CO_2 incubated 144 h at 7°C.....53

Appendix

1A Colony forming units present in Tryptic Soy Broth incubated for 18 to 24 h at 25°C, when absorbance at 420 nm is .3.....63

LIST OF FIGURES

Figure — Page

Article

1 Diagram of the growth flask used to evaluate bacterial growth in refrigerated milk held under atmospheres containing CO_2.....54

2 Growth of isolate 11p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.....55

3 Growth of isolate 17p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.....56

4 Aerobic plate counts in Raw Milk-1 with and without added CO_2 incubated for 144 h at 7°C.....57

5 Aerobic plate counts in Raw Milk-2 with and without added CO_2 incubated for 144 h at 7°C.....58

Appendices

1A Typical chromatogram for CO_2 analyses of milk from growth flasks.....64

1B Growth of isolate 1p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.....66

2B Growth of isolate 2p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.....67

3B Growth of isolate 12p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.....68

LIST OF FIGURES
(continued)

4B Aerobic plate counts in Raw Milk-3 with and without added CO_2 incubated for 144 h at 7°C.....69

5B Aerobic plate counts in Raw Milk-4 with and without added CO_2 incubated for 144 h at 7°C.....70

INTRODUCTION

The advent of on-farm refrigerated storage for raw milk in the late 1940's and its subsequent adoption by North American dairyman during the next 20 years essentially eliminated spoilage of milk by lactic acid bacteria. However, refrigeration does not prevent growth of all microorganisms present in raw milk (9, 33, 51, 52, 54, 55). Many microorganisms capable of growth at refrigeration temperatures, termed psychrotrophic bacteria, are present as normal contaminants in raw milk supplies. For purposes of clarity, psychrotrophic microorganisms have been defined as microorganisms capable of growth at 7°C or below regardless of their optimum growth temperature (9).

Milk may be held under refrigeration for 5 days or longer before processing, or pasteurization for fluid consumption. This delay in processing results from a combination of events which include alternate day pickup of bulk milk from the farm, long distance hauling of milk, and shorter work weeks for processing plants. Extended refrigerated storage of raw milk selects for growth of psychrotrophic bacteria. Presence of these bacteria decreases milk quality by producing enzymes that degrade various components of milk (1, 9, 15, 28, 33, 37, 49, 52, 54, 55). Although the majority of psychrotrophic bacteria are killed by pasteurization, their proteolytic and

lipolytic enzymes often survive (9, 15, 28). These heat-stable enzymes continue to diminish the quality of milk or other dairy products after pasteurization.

Growth and metabolic activity of psychrotrophic bacteria in refrigerated raw milk is one of the most significant quality problems facing the dairy industry today (9). As a result of this problem, novel methods for controlling psychrotrophic bacterial growth are receiving attention from researchers and industry (6, 7, 8, 12, 13, 19, 20, 23, 24, 27, 29, 31, 36, 38, 40, 45, 47, 48, 58, 59). One attractive method proposed for controlling growth of psychrotrophic bacteria involves applying low levels (10-30mM) of carbon dioxide (CO_2) to refrigerated milk. Results of preliminary studies (12, 27, 29, 47, 48) indicate CO_2 is an effective inhibitor of psychrotrophic bacterial growth in refrigerated raw milk.

The purpose of the research presented in this thesis was to evaluate the effectiveness of CO_2 as a growth inhibitor of selected pure cultures of proteolytic psychrotrophs. A method for studying growth of psychrotrophs in milk treated with CO_2 was developed. In addition, a gas chromatographic method was devised for quantifying CO_2 levels in milk.

LITERATURE REVIEW

Extended refrigerated storage during all aspects of milk handling accentuates the problem of psychrotrophic bacterial growth in refrigerated raw milk. Psychrotrophs and their enzymes reduce the quality of milk and manufactured products. Controlling growth of psychrotrophic bacteria in milk would have considerable benefit to the dairy industry. This review discusses psychrotrophic bacterial growth in refrigerated raw milk in terms of the following: (1) incidence of psychrotrophic contamination in raw milk supplies, (2) genera of bacteria involved and defects they produce, and (3) methods of controlling psychrotrophic bacterial growth with emphasis on use of CO_2.

Incidence of Psychrotrophic Bacteria in Refrigerated Raw Milk

Psychrotrophic bacteria enter raw milk from a variety of sources. These include soil, water, air, vegetation, feed, and feces (9, 33, 54, 55,). Because of the variety of sources from which psychrotrophs enter milk, the kinds of organisms and extent of contamination can vary greatly (9, 33, 54, 55). Factors influencing levels of psychrotrophic contamination include on-farm sanitary practices, season of the year, and temperature and duration of refrigerated

storage (9, 33, 54, 55).

Sanitation and milking practices on-farm are perhaps the most important factors affecting the initial levels of psychrotrophic bacterial contamination in raw milk. Thomas and Thomas (54) reported that milk produced under sanitary conditions often contains less than 1,000 CFU/ml immediately after machine milking. This supports results obtained by Tatini et al. (50) in a study evaluating the quality of bulk collected milk in Minnesota. They reported 63% of milk samples examined had psychrotrophic bacterial counts of less than 3,000 CFU/ml. In contrast, milk produced under unsanitary conditions may have psychrotrophic counts greater than 10,000 CFU/ml (54).

Sanitary practices also influence the proportion of initial bacterial flora that consists of psychrotrophic bacteria. Milk produced under sanitary conditions often contains less than 10% of the initial bacterial flora as psychrotrophs; while milk produced under unsanitary conditions may contain more than 75% of the initial bacterial flora as psychrotrophs (9, 54). In addition, to having higher levels of initial contamination, milk produced under unsanitary conditions often shows a rapid increase in numbers of psychrotrophic bacteria during refrigerated storage (9, 54). Rapid increases in psychrotrophs result from the presence of actively multiplying psychrotrophic organisms as a component of the initial flora, rather than

from higher levels of initial contamination (9, 34, 5 , 55).

Seasonal factors also determine psychrotrophic bacterial contamination in refrigerated raw milk. Thomas and Thomas (54), Erdman and Thornton (14), and Marth and Frazier (30) noted numbers of psychrotrophs are likely to be higher in summer than in winter because optimum growth temperatures for many psychrotrophic bacteria (20-30°C) are reached in poorly cleaned milk tanks in summer. This leads to higher psychrotrophic bacterial populations on milk contact surfaces, resulting in higher levels of psychrotrophs in milk. Audrey and Frazier (2) reported that counts of psychrotrophic bacteria increased approximately seven-fold when cows were switched from dry feed to pasture in the spring. In contrast, Desai and Claydon (11) reported slightly higher psychrotrophic counts in winter than in summer for bulk tank milk in Kansas. They attributed increased contamination to poor sanitation practices during colder months.

Storage temperature and initial level of psychrotrophic bacterial contamination determine the length of time raw milk can be stored without loss of quality. Thomas et al. (53) reported psychrotrophic bacterial counts in milk ranging from 0 to 13,000/ml 3 h after milking; and counts of 0 to 29 million, in the same milks, after 72 h of storage at 3-5°C. In general, decreasing storage temperatures of raw milk decreased rates of psychrotrophic bacterial growth (9).

Genera of Psychrotrophs Commonly Isolated from Raw Milk

Gram-Negative Bacteria. Cousin (9), Mikolajcik (33, 34, 35), Thomas (52), Thomas et al. (53, 54,) and Thomas and Thomas (55) in reviews on bacteria in milk and dairy products indicate psychrotrophic bacteria which are gram-negative, aerobic, asporogenous, catalase positive, and rod-shaped dominate the flora of raw milk. Members of the genus Pseudomonas are the most frequently isolated psychrotrophs, with members of the genera Alcaligenes, Flavobacterium, and Enterobacter present consistently but in smaller numbers. Strains of Pseudomonas fluorescens are the dominant psychrotrophic contaminants isolated from raw milk (9). Genera of gram-negative bacteria which have been isolated from refrigerated raw milk include Acinetobacter, Aeromonas, Alcaligenes, Chromobacterium, Citrobacter, Cytophaga, Enterobacter, Esherichia, Flavobacterium, Klebsiella, Pseudomonas, Serratia, and, Vibrio (9).

Gram-Positive Bacteria. Although early investigators were reluctant to include gram-positive microorganisms as psychrotrophs (9, 57), several genera of gram-positive organisms are frequently isolated from raw milk. These include Micrococcus, Bacillus, and Arthrobacter (9, 33).

Bacillus is the most important genus of gram-positive, psychrotrophic contamination because some species form spores which survive pasteurization and germinate at refrigeration temperatures (9, 33). However, spores of typical psychrotrophs require 8-14 days for germination and vegetative cells of these bacteria have generation times of about 24 h at 7°C (54, 55). Thus, spoilage of milk and dairy products by gram-positive psychrotrophs is slow and requires prolonged storage (8 to 20 days) under refrigeration (9). The importance of gram-positive psychrotrophic bacteria, particularly sporeformers, in refrigerated raw milk is uncertain and their significance should be evaluated for each product manufactured from contaminated milk.

Organisms of Public Health Significance. Cousin, in her 1982 review (9), summarized the information on milkborne psychrotrophic pathogens. She stated "Growth of psychrotrophs in foods usually does not result in illness since organisms able to cause foodborne illness rarely grow below 10°C and seldom below 3.3°C." Psychrotrophic pathogens of concern to the dairy industry include Bacillus cereus and Yersinia enterocolitica (9, 35). Yersinia enterocolitica is killed by pasteurization, and also during manufacture of both Cheddar and Italian-type cheeses if acid development is normal (46). Thus, Y. enterocolitica is only

a problem if post-pasteurization contamination results. The significance of B. cereus to the dairy industry needs to be determined through further research.

Defects Produced by Psychrotrophic Bacteria in Milk

The primary factors limiting shelf-life of refrigerated milk are growth and metabolic activity of psychrotrophic bacteria (9). Flavor defects produced by psychrotrophs in milk include the following: fruity and rancid (most common), putrid, potato, cheesy, bitter, unclean, soapy, and fishy (9, 33, 54, 55). Overcast (39) stated that development of off-flavors by psychrotrophic bacteria in milk occurred in three stages. These were (1) lack of freshness, (2) milk became stale, and (3) development of rancid, bitter, and fruity flavors.

Flavor defects produced by psychrotrophs in milk are caused by enzymes from psychrotrophs which act upon milk components. Proteases and lipases degrade milk proteins and lipids resulting in flavor and quality defects (9, 15, 28, 32, 34, 37, 49). Many of these proteolytic and lipolytic enzymes are heat-stable, and continue to diminish the quality of milk following pasteurization (9, 15, 28).

Numbers of psychrotrophs present when organoleptic defects are first detectable ranges from 5 to 200 million

(9). However, heat stablity of many proteolytic and lipolytic bacterial enzymes means bacterial populations less than those causing detectable defects can cause flavor defects during product storage following heat treatment. This is evidenced by gelation of milk subjected to ultra-high temperature (UHT) treatment (1). Additionally, development of a bitter flavor and decreased yield can occur in cheese made from milk contaminated with psychrotrophs (15, 28, 37, 49).

Methods Proposed to Control Growth of Psychrotrophic Bacteria

Psychrotrophic bacteria in refrigerated raw milk represent a significant problem, and thus many methods have been proposed to control their growth. These methods include improving sanitary practices (9, 15, 34), utilizing lower storage temperatures (9, 15, 34), thermization (8, 13, 20, 58, 59), pre-culturing of milk with lactic acid bacteria (19, 23, 45), activation of the lactoperoxidase system (5, 7, 24), and addition of various food additives (10, 12, 19, 27, 29, 38, 47, 48). Each of these methods will be discussed briefly in terms of practicality, effectiveness, advantages, and disadvantages.

Sanitation. Effective sanitation reduces numbers of

psychrotrophic bacteria present in raw milk supplies (9, 34, 54). However, the wide variety of sources for psychrotrophic bacterial contamination makes elimination of these organisms an impractical goal (9). The main advantage from controlling psychrotrophic contamination by sanitation is all aspects of dairy operations will benefit. This includes herd health, herd milk average, and milk quality indicators such as coliform count, standard plate count, and somatic cell count.

Refrigeration. Lowering the temperature at which milk is stored decreases growth rates of psychrotrophic bacteria (9). Storage at temperatures between 3-4°C allows raw milk to maintain acceptable microbiological quality for about 72 h (15). Storage of raw milk at lower temperatures results in an extension of storage life as determined by monitoring changes in the psychrotrophic population (9, 16). However, storage of raw milk below 2°C results in destabilization of caseins, which changes processing characteristics (43). Thus, cooling is an effective means of controlling psychrotrophic bacterial growth in milk, but excess cooling will diminish its quality.

Thermization. Thermization, or heat-treatment of milk at the farm, is an effective means of reducing numbers of psychrotrophic bacteria in a raw milk supply (8, 20, 58,

59). The process involves heating milk to 64-73.9°C for 10-15s (8, 15, 20, 58). Specific time/temperature combinations used for thermizing milk include 74°C for 10 s (13, 58, 59), 65°C for 15 s (8), and 65°C for 10 s (20). Thermization reduces microbial numbers and induces lag phase in the remaining population (15). Zall and Chen (58) reported milk heated to 73.9°C for 10 s immediately after being drawn from the cow and then cooled to 3 ± 1°C maintained satisfactory quality for 7 days of storage. Dzurec and Zall (13) reported thermization 3 days prior to manufacture of cottage cheese resulted in significant yield increases which were probably due to association of whey proteins and casein micelles. Coghill et al. (8) reported processing characteristics of milk were unaffected by thermization.

The possibility of coupling ultra-filtration or reverse osmosis with thermization on large scale dairy operations makes thermization an attractive means of controlling psychrotrophic bacterial growth in some situations (58). However, there are two major drawbacks to use of thermization. Firstly, thermization is a destructive process, i.e., the initial bacterial contamination is reduced; therefore, monitoring of sanitation practices on-farm would be difficult. Secondly, while thermization may prove worthwhile on large dairy farms, the large initial capital investment for some type of heat exchanger might

prevent use of this technology by small to average size producers.

Pre-culturing of Milk with Lactic Acid Bacteria. Several genera of lactic acid bacteria exert bacteriostatic activity against psychrotrophs (18, 19, 23, 31, 45). The primary cause of this antagonistic activity is thought to be production of hydrogen peroxide (H_2O_2). That H_2O_2 is the primary bacteriostatic agent is supported in the following two ways: first, addition of catalase to milk negates the antimicrobial influence resulting from the presence of lactic acid bacteria (18, 23, 45); and second, there is a strong correlation between amount of H_2O_2 produced by lactics and degree of inhibition observed (19).

The ability of lactic acid bacteria to produce H_2O_2 varies with genera and within species. Strains of Leuconostoc cremoris (23) and some species of Lactobacillus (19, 31) notably L. bulgaricus and L. lactis have been used effectively as inhibitors of psychrotrophs. Gilliland and Ewell (19) demonstrated that combinations of L. lactis and potassium sorbate were more effective than either treatment alone in retarding growth of psychrotrophic bacteria in raw milk. They postulated that potassium sorbate inhibited catalase activity in milk rendering the H_2O_2 produced more effective.

Slight yield increases and improved body/texture scores

for cottage and Cheddar cheeses have been reported when raw milk was pre-cultured with lactic acid bacteria (15). Additionally, shelf-life of pasteurized milk was extended by pre-culturing raw milk. However, two major problems exist when considering pre-culturing of milk as a means to control psychrotrophic bacterial growth. First, the bacterial load including normal flora and added lactics will exceed current legal limits for raw milk, and second, any temperature abuse during storage could lead to rapid souring and possibly production of curd.

The Food and Drug Administrtion (FDA) recently approved inoculation of raw milk for cheese manufacture with lactic acid bacteria (3). However, the FDA stipulates pre-cultured milk, prior to addition of lactics, is subject to the requirements imposed by the Grade A Pasteurized Milk Ordinance. Field studies conducted in manufacturing plants which utilize this system will provide data useful in evaluating the effectivness of pre-culturing to control psychrotrophic bacterial growth.

The Lactoperoxidase/Thiocyanate/Peroxide System. Bacteriocidal properties of the lactoperoxidase/thiocyanate/hydrogen peroxide system (LPS) in milk are well established (5, 6, 7, 24, 42). In order for the LPS to function, lactoperoxidase (LP), thiocyanate (SCN^-) and H_2O_2 must be present in milk. Milk contains

approximately 30 ug LP/ml which is sufficient for bacteriocidal activity (7) . Concentrations of SCN^- in milk depend upon the diet of the cow and range from .017 to .26 mM (7). Hydrogen peroxide, the third component of the LPS, is not normally present at levels sufficient for bacteriocidal activity (approximately .3 mM) (7). However, two methods have been proposed for raising the H_2O_2 content to required levels. The first is simply to add sufficent dilute H_2O_2 (24). The second method involves adding glucose and glucose oxidase to milk in order to generate H_2O_2 *in situ* (7). Both of these methods produce acceptable bacteriocidal activity.

The mode of action of the LPS is not fully understood. Aune and Thomas (4) reported hypothiocyanite ($OSCN^-$) accumulated during LP catalyzed oxidation of SCN^- and postulated $OSCN^-$ is responsible for the bacteriocidal activity of the LPS. Alternatively, Hogg and Jago (22) postulated that antimicrobial activity of the LPS is due either to cyanosulfurous acid (HO_2SCN) or cyanosulfuric acid (HO_3SCN). Bjorck and Claesson (6) reported addition of chemically prepared $OSCN^-$ to milk did not have the same bacteriocidal activity as the LPS system. This supports the hypothesis that unstable intermediates of the LPS (HO_2SCN or HO_3SCN) are responsible for its biological activity.

Bjorck (5) was able to store milk for five days at 5^oC with no significant increase in psychrotrophs. Kamau and

Kroger (24) reported that at temperatures above those normal for refrigeration, 14, 20, and 30°C, antimicrobial activity was observed. However, antimicrobial activity was greater at 14°C than at either of the higher temperatures.

An advantage of the LPS is activation does not adversely influence processing characteristics of milk. Additionally, any residual H_2O_2 can be easily removed by addition of catalase. A disadvantage of the LPS is that the process is bacteriocidal, thus monitoring sanitary conditions of milk production is difficult. Also, there is a perception among dairymen and others that if a little is good a lot is better and addition of large amounts of H_2O_2 to milk would diminish its quality.

Addition of Food Additives. Many food additives have been proposed for use as inhibitors of psychrotrophs in milk (36, 38). These include chlortetracycline, nisin, bacitracin, chloramphenicol, lysozyme, ethylenediaminetetraacetic acid, nitrofurazone, propyl-p-hydroxybenzoate, sodium benzoate, and potassium sorbate (38). Carbon dioxide (CO_2) can also be considered a food additive, and will be discussed in a following section.

Of the many additives investigated, only potassium sorbate has shown effectiveness at concentrations not causing defects in milk. Moustafa and Collins (38) showed potassium sorbate could retard growth of Pseudomonas fragi

in skim milk and half-and-half acidified to pH 5.2. More recently, Mistry and Kosikowski (36) determined .075% potassium sorbate added to pasteurized milk significantly reduced growth of psychrotrophic bacteria. These workers also found combinations of potassium sorbate and H_2O_2 very effective inhibitors of bacterial growth. A disadvantage to use of sorbate as a preservative for raw milk is the sweet flavor sorbate imparts to milk when used at levels above .2%.

Addition of Carbon Dioxide.

Carbon dioxide is known to stimulate (44, 56) and inhibit (10, 12, 16, 17, 26, 27, 29, 41, 47) bacterial growth. Factors affecting the biological activity of CO_2 include the following: concentration of CO_2; nutrient composition, pH, ionic strength, and water activity of the growth medium; and temperature of incubation (10). Carbon dioxides bacteriostatic properties have been exploited to extend shelf-life of a number of commodities. Fruits, vegetables, eggs, carbonated beverages, pork, poultry, beef, and seafoods have been preserved with CO_2 (10). Although the bacteriostatic action of CO_2 has been studied for over 100 years, its mode of action is not completely understood. For a thorough review of possible modes of action, see Daniels et al. (10).

The psychrotrophic spoilage flora of raw milk is dominated by species of Pseudomonas. The effects of CO_2 on growth and metabolic activity of strains of Pseudomonas have been reported (16, 17, 25, 26, 27, 47).

In 1967, King and Nagel (25) studied the effects of CO_2 on growth of Pseudomonas aeruginosa, while holding temperature, oxygen tension, pH, and ionic strength of the medium constant. These workers reported a linear relationship existed between generation time of P. aeruginosa and CO_2 content of atmospheres over a wide range of CO_2 concentrations (0-70% CO_2). When concentrations of atmospheric CO_2 were 50%, the growth rate of P. aeruginosa was significantly lower than the same organism growing in air. In an extension of their work, King and Nagel (26) determined the influence of CO_2 upon metabolism of P. aeruginosa growing on several substrates in atmospheres containing air and CO_2 (50% v/v). These experiments demonstrated the bacteriostatic effect of CO_2 is not substrate dependent, i.e. bacteriostatic action was observed regardless of growth medium. However, degree of inhibition by CO_2 did vary with differing substrates. During these experiments an effort was made to determine if CO_2 interfered with a particular enzyme by monitoring for a buildup of metabolic intermediates. No buildup of intermediates was observed, but work with purified enzymes

demonstrated that CO_2 inhibited isocitrate dehydrogenase and malate dehydrogenase activity. King and Nagel postulated CO_2 exerts a mass action effect upon rates of certain enzymatic decarboxylations, which slows the overall metabolic rate.

Gill and Tan (16) reported the effect of CO_2 on growth of P. fluorescens PDD 3513. At low levels (100 mm Hg) in minimal medium, CO_2 enhanced growth of P. fluorescens; but at higher concentrations growth was inhibited. In a complex medium, all concentrations of CO_2 used (100 to 400 mm Hg CO_2) resulted in inhibition of growth. Gill and Tan (16) also showed that decreasing the temperature of incubation increased inhibition by CO_2. At no temperature or CO_2 concentration did sterilization result.

King and Mabbitt (27) studied growth of P. fluorescens (NCDO 2085) in both nutrient broth and skim milk under atmospheres containing CO_2. These workers concluded the main effect of CO_2 was to increase the lag phase of growth. This conclusion is not supported by the work of King and Nagel (25, 26), which indicated an extension of lag phase growth, and decreased growth rates during exponential growth. Concentration of CO_2, growth phase of inoculum at time of treatment with CO_2, temperature of incubation, and composition of the growth medium influence growth of psychrotrophs and makes comparison of inhibition data from different laboratories difficult.

The preceding research reports indicate CO_2 is an effective inhibitor of psychrotrophic organisms that contaminate raw milk. Following is a presentation of reports dealing with the bacteriostatic action of CO_2 in refrigerated milk.

Use of carbon dioxide as a Preservative in Milk. The idea of using CO_2 as a preservative for milk and dairy products is not new. Valley and Rettger (56) in 1927 reported an unsuccessful attempt to inhibit growth of mesophilic spoilage organisms in raw milk and ice cream by applying CO_2. Their success was limited because they used temperatures (21-25°C) above those currently used to store milk (3-4°C). Also, these workers studied mesophilic bacteria, not psychrotrophic bacteria. Since that time, bacteriostatic activity of CO_2 has been shown to be enhanced at low (refrigeration) temperatures (16, 17). Prucha et al. (41) in 1931 performed an exhaustive study concerning effects of carbonation on bacterial content and keeping quality of various dairy products. These workers concluded there was no economic advantage to be gained by treating dairy products with CO_2. However, they reported raw milk, if refrigerated, would maintain its bacteriological quality longer when treated with CO_2 than milk refrigerated but not treated with CO_2. It is possible Prucha et al. did not think this extension in keeping quality of refrigerated raw

milk was important because the refrigerated bulk milk handling system, currently used on dairy farms in the United States, was not yet in place.

In 1982, several studies concerning use of CO_2 to control psychrotrophic spoilage organisms in refrigerated raw milk were published (27, 29, 47). Results reported were similar to those obtained by Prucha et al. (41) but objectives of experiments had changed. Prucha et al. (41) wanted to preserve raw milk for extended periods, perhaps without refrigeration; a function CO_2 is not capable of performing. The objective of more recent research has been extending holding time for raw milk under refrigeration prior to processing. This is a function which CO_2 performs very well.

King and Mabbitt (27) report refrigerated raw milk treated with 10 to 30 mM CO_2 could be held for 1 to 3 days longer than untreated milk prior to reaching an arbitrary quality cut-off point (10^6 CFU/ml). These workers showed the bacteriostatic action of CO_2 in milk was not due to decrease in pH, or to displacement of dissolved oxygen. Shipe et al. (47) reported raw milk treated with CO_2 remained acceptable longer than untreated milk. In the work of Shipe et al., raw milk treated with CO_2 (165 to 575 ppm) supported approximately 50% less bacterial growth than did untreated milk after 48 hours of incubation at 6.7° C. Shipe et al. (47) also performed experiments with freshly

pasteurized milk inoculated with P. putida and reported growth of this organism at 6.7°C could be inhibited up to 99% by treatment with CO_2. In addition, Shipe et al. showed other common spoilage organisms including species of Pseudomonas and Enterobacter were inhibited by treatment with CO_2.

In a continuation of the work of Shipe et al. (47), Duthie et al. (12) treated pasteurized milk with CO_2. She reported significant shelf-life extensions and lower psychrotrophic counts than were observed for ungassed controls after 7 days of refrigerated storage. Upon evaluation, no differences in flavor due to treatment with CO_2 were detected by trained or untrained panelists. It is difficult to relate these flavor studies to results from other research because exact concentrations of CO_2 present at the time of organoleptic analyses were not reported.

Skudra (48) reported total bacterial counts decreased initially when CO_2 was applied. He attributed decreases in total counts to decreases in numbers of proteolytic and lipolytic bacteria present. Bacteriocidal activity related to CO_2 had not been reported previously. However, high storage temperature (18° C) and high concentration of CO_2 (8,000-14,500 ppm CO_2) used in this research makes comparison of this work with previous studies conducted in milk difficult. Skudra did report that Tvorog could be manufactured satisfactorily from treated milk without

removing CO_2.

Carbon dioxide is known to stimulate growth of certain bacteria (44, 56). Thus, a potential criticism for use of CO_2 to control growth of psychrotrophs in refrigerated milk is CO_2 will select for growth of other organisms including potential pathogens. No information is available concerning effects of CO_2 on growth of pathogenic bacteria in refrigerated milk. But, as Cousin points out in her review (9), refrigeration controls growth of most pathogenic bacteria. One report concerning the effects of CO_2 on germination characteristics of Bacillus spp. (21) indicated CO_2 in the medium stimulated germination of B. cereus (about 20% stimulation) and several other species of Bacillus. However, this research utilized saturating concentrations of CO_2. In practice, saturating levels of CO_2 would render milk unacceptable for consumption by destabilizing milk protein (27, 29).

A major advantage when considering use of CO_2 as a preservative in milk is ease of removal. As King and Mabbitt (27) point out, warming milk under a gentle vacuum (vacreation) or gentle agitation at ambient temperature would remove all CO_2. In addition, milk as it is drawn from the cow, contains about 5% CO_2 per volume of milk, thus the addition of CO_2 would only involve adding back a normal component of milk. Furthermore, CO_2 at concentrations below 30 mM, does not influence processing characteristics of milk

(29).

Carbon dioxide inhibits the organisms found most often in refrigerated raw milk. In addition, preliminary studies using naturally contaminated raw milk indicate treatment with CO_2 extends storage-life. However, little information is available regarding the influence of CO_2 on pure cultures of bacteria growing in refrigerated milk. Further research is recommended to determine the influence of CO_2 on growth of pathogens and Gram-positive spore-formers in milk. Also, the effect of CO_2 on processing characeristics of milk must be determined. Only then can commercial application of CO_2 treatment be recommended for fluid raw milk.

The present research was undertaken to determine what effect CO_2 had upon growth rates of pure cultures of proteolytic psychrotrophic bacteria in refrigerated milk.

REFERENCES

1 Adams, D. M., J. T. Barach, and M. L. Speck. 1976. Effect of psychrotrophic bacteria on milk proteins and stability of milk proteins to ultrahigh temperature treatment. J. Dairy Sci. 59:823.

2 Audrey, J. Jr., and W. C. Frazier. 1959. Psychrophiles in milk held two days in farm bulk cooling tanks. J. Dairy Sci. 42:1781.

3 Anonymous. 1985. FDA: Pre-culturing milk to control psychrotrophic development okay. National Dairy News 5(20):11.

4 Aune, T. M., and E. L. Thomas. 1977. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. Eur. J. Biochem. 80:209.

5 Bjorck, L. 1978. Antibacterial effect of the lactoperoxidase system on psychrotrophic bacteria in milk. J. Dairy Res. 45:109.

6 Bjorck, L., and O. Claesson. 1980. Correlation between concentration of hypothiocyanate and antibacterial effect of the lactoperoxidase system against Escherichia coli. J. Dairy Sci. 63:919.

7 Bjorck, L., C. G. Rosen, V. Marshall, and B. Reiter. 1975. Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. Appl. Microbiol. 30:199.

8 Coghill, D. M., I. D. Mutzelburg, and S. H. Birch. 1982. Effect of thermization on the bacteriological and chemical quality of milk. Aust. J. Dairy Technol. 37:48.

9 Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A Review. J. Food Prot. 45:172.

10 Daniels, J. A., R. Krishnamurthi, and S. S. H. Rizvi. 1985. A review of the effects of carbon dioxide on microbial growth and food quality. J. Food Prot. 48:532.

11 Desai, M. N., and T. Claydon. 1964. Preliminary incubation of raw milk samples as an aid in evaluating bacteriological quality. J. Milk & Food Technol. 27:333.

12 Duthie, C. M., W. F. Shipe, and J. H. Hotchkiss. 1985. Effect of low level carbonation on the keeping quality of processed milk. J. Dairy Sci. 68(suppl. 1):69 (Abstr.).

13 Dzurec, D. J., and R. R. Zall. 1982. Effect of on-farm heating and storage of milk on cottage cheese yield. J. Dairy Sci. 65:2296.

14 Erdman, J. E., and H. R. Thornton. 1951. Psychrophilic bacteria in Edmonton milk and cream. Can. J. Technol. 29:232.

15 Fairbairn, D. J., and B. A. Law. 1986. Proteinases of psychrotrophic bacteria: Their production, properties, effects, and control. J. Dairy Res. 53:139.

16 Gill, C. O., and K. H. Tan. 1979. Effect of carbon dioxide on growth of Pseudomonas fluorescens. Appl. Environ. Microbiol. 38:237.

17 Gill, C. O., and K. H. Tan. 1980. Effect of carbon dioxide on growth of meat spoilage bacteria. Appl. Environ. Microbiol. 39:317.

18 Gilliland, S. E., and M. L. Speck. 1975. Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. J. Food Sci. 40:903.

19 Gilliland, S. E., and H. R. Ewell. 1983. Influence of combinations of Lactobacillus lactis and potassium sorbate on growth of psychrotrophs in raw milk. J. Dairy Sci. 66:974.

20 Gilmour, A., R. S. Macelhinney, D. E. Johnston, and R. J. Murphy. 1981. Thermisation of milk some microbiological aspects. Milchwissenschaft 36:457.

21 Guirguis, A. H., M. W. Griffiths, and D. D. Muir. 1984. Spore-forming bacteria in milk. II. Effect of carbon dioxide addition on heat activation of spores of Bacillus species. Milchwissenschaft 39:144.

22 Hogg, D. McC., and G. R. Jago. 1970. The antibacterial action of lactoperoxidase. Biochem. J. 117:779.

23 Juffs, H. S., and F. J. Babel. 1975. Inhibition of psychrotrophic bacteria by lactic cultures in milk stored at low temperature. J. Dairy Sci. 58:1612.

24 Kamau, D. N., and M. Kroger. 1984. Preservation of milk by treatment with hydrogen peroxide and by activation of the lactoperoxidase (LP) system. Milchwissenschaft 39:658.

25 King, A. D., and C. W. Nagel. 1967. Growth inhibition of Pseudomonas by carbon dioxide. J. Food Sci. 32:575.

26 King, A. D., and C. W. Nagel. 1975. Influence of carbon dioxide upon the metabolism of Pseudomonas aeruginosa. J. Food Sci. 40:362.

27 King, J. S., and L. A. Mabbitt. 1982. Preservation of raw milk by the addition of carbon dioxide. J. Dairy Res. 49:439.

28 Law, B. A. 1979. Reviews of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46:573.

29 Mabbitt, L. A. 1982. Preservation of refrigerated milk. Kiel. Milchwirtsch. Forschungsber. 34:28.

30 Marth, E. H., and W. C. Frazier. 1957. Bacteriology of milk held at farm bulk cooling tank temperatures. J. Milk & Food Technol. 20:72.

31 Martin, D. R., and S. E. Gilliland. 1980. Inhibition of psychrotrophic bacteria in refrigerated milk by lactobacilli isolated from yogurt. J. Food Prot. 43:675.

32 McKellar, R. C. 1981. Development of off-flavors in ultra-high temperature pasteurized milk as a function of proteolysis. J. Dairy Sci. 64:2138.

33 Mikolajcik, E. M. 1979. Psychrotrophic bacteria and dairy product quality. 1. Major organisms involved and defects produced. Cult. Dairy Prod. J. 14:6.

34 Mikolajcik, E. M. 1980. Psychrotrophic bacteria and dairy product quality. 2. Detection of problem organisms and their control. Cult. Dairy Prod. J. 15:5.

35 Mikolajcik, E. M. 1980. Psychrotrophic bacteria and dairy product quality. 3. Organisms of public health importance in fermented dairy foods. Cult. Dairy Prod. J. 15:14.

36 Mistry, V. V., and F. V. Kosikowski. 1985. Influence of potassium sorbate and hydrogen peroxide on psychrotrophic bacteria in milk. J. Dairy Sci. 68:605.

37 Mohamed, F. O., and R. Bassette. 1979. Quality and yield of cottage cheese influenced by psychrotrophic microorganisms in milk. J. Dairy Sci. 62:222.

38 Moustafa, H. H., and E. B. Collins. 1969. Effects of selected food additives on growth of Pseudomonas fragi. J. Dairy Sci. 52:335.

39 Overcast, W. W. 1967. Extending shelf-life by controlling psychrophiles. Amer. Dairy Rev. 29:42.

40 Price, R. J., and J. S. Lee. 1970. Inhibition of Pseudomonas species by hydrogen peroxide producing lactobacilli. J. Milk Food Technol. 33:13.

41 Prucha, M. J., J. M. Brannon, and H. A. Ruehe. 1931. Effect of carbonation on bacterial content and keeping quality of dairy products. Univ. of IL Ag. Exp. Station Bull. 368.

42 Reiter, B. 1978. Review of the progress of dairy science: Antimicrobial systems in milk. J. Dairy Res. 45:131.

43 Reimerdes, E. H. 1982. In Developments in Dairy Chemistry-I. Proteins. (Ed. P. F. Fox) London: Applied Science Publishers.

44 Repaske, R., and M. A. Clayton. 1978. Control of Escherichia coli growth by CO_2. J. Bacteriol. 135:1162.

45 Ross, G. D. 1981. The inhibition of growth of spoilage organisms in milk by Streptococcus lactis subsp. diacetylactis, Leuconostoc cremoris, and L. dextranicum. Aust. J. Dairy Technol. 36:147.

46 Schiemann, D. A. 1978. Association of Yersinia enterocolitica with the manufacture of cheese and occurrence in pasteurized milk. Appl. Environ. Microbiol. 36:274.

47 Shipe, W. F., G. F. Senyk, E. J. Adler, and R. A. Ledford. 1982. Effect of infusion of carbon dioxide on the bacterial growth in fluid milk. J. Dairy Sci. 65(Suppl. 1):77 (Abstr.).

48 Skudra, L. A. 1983. Effect of carbon dioxide on the microflora of milk. [Trudy, Latviiskaya Ordena Trudovogo Krasnogo Znameni Sel'skohozyaistvennaaya Akademiya] (1982) No. 196, 3-15 (Dairy Sci. Abstr. 45:268).

49 Stofer, W., and C. L. Hicks. 1983. Pernicious psychrophiles - Their effect on cheese yield and composition. Cultured Dairy Prod. J. 18:11.

50 Tatini, S. R., R. Dabbah, and J. C. Olson. 1965. Interrelationships between some bacteriological methods used for the examination of farm bulk tank milk supplies. J. Milk & Food Technol. 28:368.

51 Thomas, S. B. 1958. Psychrophilic microorganisms in milk and dairy products. Part I. Dairy Sci. Abstr. 20:335.

52 Thomas, S. B. 1958. Psychrophilic microorganisms in milk and dairy products. Part II. Dairy Sci. Abstr. 20:449.

53 Thomas, S. B., R. G. Druce, and M. Jones. 1966. The significance of psychrotrophic bacteria in raw milk. Dairy Ind. 31:27.

54 Thomas, S. B., and B. F. Thomas. 1973. Psychrotrophic bacteria in bulk collected raw milk. Part 1. Dairy Ind. 38:11.

55 Thomas, S. B., and B. F. Thomas. 1973. Psychrotrophic bacteria in bulk collected raw milk. Part 2. Dairy Ind. 38:61.

56 Valley, G., and L. F. Rettger. 1927. The influence of carbon dioxide on bacteria. J. Bacteriol. 14:101.

57 Witter, L. D. 1961. Psychrophilic bacteria. A Review. J. Dairy Sci. 44:983.

58 Zall, R. R., and J. H. Chen. 1981. Heating and storing milk on dairy farms before pasteurization in milk plants. J. Dairy Sci. 64:1540.

59 Zall, R. R., J. H. Chen, and S. C. Murphy. 1982. Effects of heating and storing milk in milk plants before pasteurization. Cult. Dairy Prod. J. 17:5.

Running Head: GROWTH INHIBITION BY CARBON DIOXIDE

Inhibition of Psychrotrophic Bacterial Growth in Refrigerated Milk by Addition of Carbon Dioxide[1]

R. F. ROBERTS[2] and G. S. TORREY

Dairy Science Department
South Dakota State University
Brookings, SD 57007

KEY WORDS: carbon dioxide, psychrotrophic bacteria, Pseudomonas, milk, inhibition, preservation

---

[1] Published with approval of the Director of the South Dakota Agriculture Experiment Station as Publication No. ---- of the Journal Series.

[2] Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108.

ABSTRACT

Treatment of refrigerated milk with 20 to 30 mM CO_2 was evaluated as a method for extending storage-life by inhibiting growth of psychrotrophic bacteria. Generation times were significantly longer for each of five psychrotrophic pseudomonads isolated from milk when grown at 7°C in sterile milk treated with CO_2 than when the same bacteria were grown in ungassed sterile milk. When raw milks were stored at 7°C and treated with CO_2 the time required for plate counts to increase 10 fold was at least 24 h longer than in the same milks left untreated. Numbers of coliforms, psychrotrophs, and anaerobes (facultative and obligate) were significantly lower in raw milks treated with CO_2 than in untreated milks after 6 days incubation at 7°C.

INTRODUCTION

The advent of on-farm refrigerated storage for raw milk in the late 1940's and its acceptance by North American dairymen during the next twenty years essentially eliminated spoilage of milk by lactic acid bacteria. However, refrigeration does not prevent growth of all microorganisms present in raw milk (3, 22). Many bacteria capable of growth at refrigeration temperatures, termed psychrotrophic

bacteria, are present as normal contaminants in raw milk. For purposes of clarity, psychrotrophic microorganisms have been defined as microorganisms capable of growth at 7°C or below regardless of their optimum growth temperature (3).

Raw milk can be held under refrigeration for up to 5 days prior to being pasteurized for fluid consumption or processed into various dairy products (3). Factors responsible for extended refrigerated storage include every-other-day pick up of refrigerated bulk collected milk, increased milk hauling distances, and shortened work weeks for processing plant personnel (3). Storage of raw milk under refrigeration selects for growth of psychrotrophic bacteria. These bacteria diminish milk quality through growth and production of enzymes that degrade various components of milk (3, 6, 13, 22). Although the majority of psychrotrophic bacteria do not survive pasteurization, some of their enzymes, including proteases and lipases, do survive and continue to diminish the quality of milk or other dairy products.

Methods for controlling psychrotrophic bacterial growth are receiving a lot of attention, because growth and activity of psychrotrophic bacteria in refrigerated raw milk is a significant quality problem for the dairy industry. One method proposed for controlling bacterial growth in refrigerated milk involves application of low levels (10-30mM) of carbon dioxide (CO_2). Results of preliminary

studies (5, 10, 14, 18, 19, 20) indicate CO_2 is an effective inhibitor of psychrotrophic bacterial growth in refrigerated raw milk. However, there is a lack of information concerning influences of CO_2 on growth of specific organisms in refrigerated milk.

The purpose of this research was to evaluate the effectiveness of CO_2 as a growth inhibitor of selected psychrotrophs in milk. Also investigated was the effect of CO_2 on normal bacterial flora of refrigerated raw milk, and a method was developed for evaluating bacterial growth in milk incubated under altered gas atmospheres.

MATERIALS AND METHODS

Isolation and Identification of Proteolytic, Psychrotrophic Bacteria

Proteolytic psychrotrophic bacteria were isolated from raw milk samples obtained from the South Dakota Dairy Laboratory by surface-plating .1 ml of an appropriate dilution on Standard Methods Caseinate Agar (SMCA) (16). Following incubation at 7°C for 10 days, colonies surrounded by a halo of precipitation were picked for identification. Isolates were characterized on the basis of Gram reaction, cellular morphology, location of flagella, catalase reaction (8), oxidase test (8), growth at 5 and 41°C, reaction on

Hugh-Leifson oxidative fermentative medium (2), casein hydrolysis (15), lipolytic activity (2), and results of the Oxi-Ferm Tube rapid identification tests (Roche Diagnostic Systems, Nutley, NJ). Five isolates were chosen for use in growth studies based upon proteolytic action on SMCA. Cultures were maintained by monthly transfer on Tryptic Soy Agar (Difco Laboratories, Detroit, MI) and were held at 7°C.

Determination of the Carbon Dioxide Content of Milk

The concentration of CO_2 present in milk samples was determined by gas liquid chromatography using a modification of the method of King and Mabbitt (9). Five milliliters of milk was transfered to a 100 ml serum bottle sealed with a Mininert^R valve (Supelco Inc., Bellefonte, PA) and acidified with 1 ml of 1 N H_2SO_4. After thorough mixing, samples were held for a minimum of 5 minutes to insure release of CO_2 from the milk. Following equilibration, .1 ml of head-space atmosphere was removed with a gas-tight syringe (Precision Sampling Corp., Baton Rouge, LA), and injected into a Varian 1520 gas-liquid chromatograph (Varian Instruments, Palo Alto, CA) equiped with a thermal conductivity detector, and a stainless steel column (2.4 m by 1.65 mm ID) packed with Porapak-Q (100-120 mesh) (Waters Assoc., Milford, MA). The detector and injector temperatures were 170 and 150°C

respectively, and the detector bridge current was 200 mA. Helium was used as the carrier gas at a flow rate of 25 cc/min. Concentration of CO_2 in milk samples were determined by relating peak heights to a standard curve for CO_2 prepared from bicarbonate solutions. A typical chromatogram for this analyses is presented in Appendix A (Figure 1A). All CO_2 analyses were in duplicate.

Growth Flasks

A 1 liter Erlenmeyer flask (Pyrex # 4985) was modified for studying growth of psychrotrophic bacteria in milk treated with CO_2 (Figure 1) Modifications included annealing the threaded portion of a 13 mm screw cap test tube 5 cm above the bottom of the flask. To facilate gassing of growth flask atmospheres, a 1 mm hole was drilled in the top of each cap, and caps were fitted with septa made from GR 2, 3 mm Silicone Sheet Stock (Supelco Inc., Bellefonte, PA). Concentrations of CO_2 in growth flasks remained constant when empty flasks were gassed with CO_2 and analyzed intermittently during a 7 day period.

Addition of Carbon Dioxide to Milks

Growth flasks containing 250 ml of milk inoculated with the appropriate isolate, or 250 ml of naturally contaminated raw milk, were treated with CO_2 by successively removing 50 cc of flask atmosphere through the sampling port in the cap of the flask and adding 50 cc of CO_2. This was repeated until approximately 60% of the atmosphere had been replaced with CO_2. Finally, 100 cc of modified atmosphere was removed and replaced with O_2 to insure similar O_2 levels in gassed and ungassed flasks. The CO_2 and O_2 used were food grade (Tri-State Welding, Watertown, SD).

Growth Studies with Pure Cultures

Inocula were prepared for each isolate, by inoculating 10 ml of Tryptic Soy Broth (TSB, Difco) and incubating the broth at 25°C for 24 h. One tenth of a milliliter of culture was transferred to 10 ml of fresh TSB and incubated for 18 h at 25°C. Before inoculating growth flasks, 1 ml of this broth was diluted to an absorbance of .3 at 420 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY). Cell numbers for each isolate at this absorbance had been determined previously Appendix A, Table 1A). The culture was then diluted to a cell concentration

of 2.5 X 10^5 CFU/ml and 1 ml of diluted broth was used to inoculate milk samples.

Raw whole milk was obtained from the South Dakota State Dairy Research and Production Unit, dispensed into sterile growth flasks (250 ml/flask), and heat-treated at 110°C for 10 min. After cooling to 7°C, flasks were inoculated.

Data for growth curves were generated by plating 1 or .1 ml of appropriate dilutions and pouring plates with Standard Methods Agar (SMA, Difco) according to standard methods (15). Plates were incubated for 48 h at 25°C prior to counting. Milks were held at 7°C and sampled twice daily during the 136 h incubation. All plate counts were in duplicate.

The pH of ungassed milk was determined initially after cooling and at the end of the experimental period. Measurements were made with an Orion Research Model 701A Digital Ion Analyzer (Orion Research, Cambridge, MA) equipped with a combination electrode (Orion # 91-02). Carbon dioxide concentrations in treated milks were determined after 18 h of incubation and at the end of the experimental period.

Growth Studies with Naturally Contaminated Raw Milk

Raw whole milk was obtained from farms in Brookings County, South Dakota. Growth studies of the natural flora in raw milk were conducted in the same manner as growth studies for pure cultures in heat-treated milks with two exceptions. First, heat-treatment was omitted for this study and second, plate counts were done daily for 6 days instead of twice daily.

In addition to daily total aerobic plate counts at 25°C, coliforms, psychrotrophs, and anaerobes were enumerated on days 1, 3, and 6. Standard methods (15) were used for coliform and psychrotrophic bacterial counts. Bacterial counts of obligate and facultative anaerobes were determined by plating appropriate dilutions on SMA supplemented with 2 g of sodium thioglycolate and 10 g of glucose/ 1. Poured plates for anaerobic counts were incubated anaerobically for 72 h at 25°C in GasPak^{R} systems (Baltimore Biological Laboratory, Cockeysville, MD).

Statistical Analysis

Growth rates of pure cultures in milk and milk treated with CO_2 were compared by performing linear regression over the linear portion of growth curves and testing for

differences between slopes (21). Populations of coliforms, psychrotrophs, and anaerobes in CO_2 treated and untreated raw milks following 6 days of incubation at 7°C were compared using analysis of variance (21).

RESULTS AND DISCUSSION

Isolation and Characterization of Psychrotrophic Bacteria

Psychrotrophic bacteria used in growth experiments were oxidase positive, Gram-negative, asporogenous rods which utilized glucose aerobically, hydrolyzed casein, and were motile by means of polar flagella (Table 1). Based on these characteristics all 5 isolates were identified as belonging to the genus Pseudomonas (12). Isolates 1p and 2p produced water soluble fluorescent pigment and were further identified as Pseudomonas fluorescens. The two fluorescent isolates differed in their ability to hydrolyze urea, reduce nitrate, and grow at 41°C. Among isolates 11p, 12p, and 17p, urea hydrolysis, nitrate reduction, and gelatin liquefaction were found to occur in different combinations. Previous researchers (3, 6, 13, 22) have reported species of the genus Pseudomonas dominate as spoilage flora of refrigerated milk. In addition, pseudomonads isolated from refrigerated milk are known to produce enzymes which

diminish the quality of milk and manufactured dairy products (3, 6, 13).

Growth Studies with Pure Cultures

Initial and final concentrations of CO_2 used in growth experiments (Table 2) indicated no loss of CO_2 throughout the 136 h experimental period. The narrow range of CO_2 concentrations, 21.5 to 24.8 mM, achieved in pure culture experiments, demonstrates gassing in the described manner resulted in uniform CO_2 concentrations. King and Mabbitt (9) attributed difficulties in maintaining CO_2 concentrations in growth vessels to incomplete sealing.

Due to the equilibrium involving dissolved CO_2, carbonic acid, and bicarbonate when CO_2 is added to an aqueous medium (4), addition of CO_2 lowers milk pH (8, 14, 19). The initial pH of milks used in growth experiments ranged from 6.59 to 6.70 (Table 3), well within the range for normal milk (1). Addition of 20 to 30 mM CO_2 to these milks resulted in final pH values near 6.2, which is similar to the decrease from the pH for normal milk reported by King and Mabbitt for CO_2 concentrations in the same range (9). Several workers have reported that the decrease in pH of milk caused by addition of CO_2 is not responsible for the bacteriostatic action observed (4, 9, 10, 11).

Treatment of milk incubated at 7°C with 20 to 30 mM CO_2

resulted in significant decreases in growth rates of psychrotrophs (Table 4). Examination of percent uninhibited growth rates (Table 4) reveals that as generation times in air decreased, degree of growth inhibition by CO_2 increased. The generation times (GT) of isolates growing in untreated milk at 7°C were similar to those reported by Gill and Tan (7) for fluorescent and non-fluorescent pseudomonads growing on meat at 3°C. Growth experiments with CO_2 were conducted at 7°C to shorten the experimental period to one week. Because the inhibitory properties of CO_2 increase with decreasing temperature (4, 7, 9), and growth rates of psychrotrophs also decrease as temperatures for growth are lowered (3), CO_2 treatment of milk held at normal refrigeration temperatures (3-4°C) should result in even greater inhibition of bacterial growth than was observed in these experiments at 7°C.

Growth data for isolates 11p and 17p growing in milk and milk treated with CO_2 are presented in this report (Figures 2 and 3) because they represent the greatest and least degree of inhibition by CO_2 of the five isolates tested. Growth curves for isolates 1p, 2p, and 12p, used to compute generation times, are not presented, but can be found in Appendix B (Figures 1B, 2B, and 3B). Linearity of growth curves during the first 48 h of the experimental period indicated cells were in the logarithmic phase of growth. Carbon dioxide reportedly prolongs the lag phase and does

not influence cells in logarithmic growth when used to preserve milk (9). Since generation times were significantly increased by treatment with CO_2 (Table 4), the influence of CO_2 on bacterial growth appears to depend upon the growth phase of cells at the time of treatment. Increased generation times, as well as increased lag phases, have been reported for organisms growing on various foods under atmospheres containing CO_2 (7, 10, 11).

Growth Studies with Raw Milk

The concentrations of CO_2 used in experiments with raw milk are presented in Table 5. These concentrations are similar to those used by previous researchers studying bacterial growth in raw milk treated with CO_2 (9, 14).

Aerobic plate count data for Raw Milk-1 (RM1), and Raw Milk-2 (RM2), Figures 4 and 5 represent the highest and lowest levels of initial microbial contamination of the four farm milks tested. Growth data for Raw Milk-3 and Raw Milk-4 can be found in Appendix B (Figures 4B and 5B). Growth of normal flora in RM1, Figure 4, represents results for manufacturing grade milk, while the growth of normal flora in RM2, Figure 5, represents results for Grade A milk produced under excellent conditions. These growth studies are similar to those described by King and Mabbitt (9). In the case of RM1, the benefits of applying CO_2 were evident

after 48 h of incubation at 7°C. If a contamination level of 10^6 CFU/ml is chosen as an end point for microbiological acceptability, then treatment of RM1 with CO_2 resulted in about 3 days extension in storage-life. Because of the lower initial level of contamination, the benefits of applying CO_2 to RM2 became apparent only after 96 h of incubation at 7°C. Neither treated nor untreated RM2 samples reached the 10^6 CFU/ml quality end point before completion of the experiment. It is apparent that treated milk will remain acceptable for an extended period of time.

During growth experiments with raw milks, numbers of various groups of microorganisms important to quality of milk for manufacturing were periodically monitored. Total aerobes (25°C), coliforms, psychrotrophs, and an estimate of the anaerobic population after 6 days of incubation at 7°C are presented in Table 6. In all bulk farm milk samples, populations of coliforms, psychrotrophs, and anaerobes were significantly lower in CO_2 treated milks than in control milks.

A potential criticism for using CO_2 as a preservative for refrigerated raw milk is that addition of CO_2 will allow anaerobic and facultatively anaerobic bacteria to grow, while at the same time inhibiting aerobic psychrotrophic spoilage bacteria. At the level of CO_2 used in these experiments, 20 to 30 mM, the stimulatory action of CO_2 reported by other workers (17, 23) towards E. coli and other

mesophilic spoilage bacteria was not observed.

Daniels et al. (4) reviewed the use of CO_2 to inhibit bacterial growth and concluded that the mode of action of CO_2 as a bacteriostatic agent is not completely understood. However, the decrease in both pH and dissolved oxygen content that occurs upon addition of CO_2 to growth media are not responsible for bacteriostatic action of CO_2 (4, 9, 10, 11). Sears and Eisenberg (18) working with a model system found ionic permeability of a model "membrane" composed of water and benzene decreased when CO_2 was introduced. They postulated this decreasing permeability stresses the system and depresses the growth rate. Other workers (7, 10, 11) believe CO_2 exerts a mass action effect against certain decarboxylating enzymes. Daniels et al. (4) have suggested dissolved CO_2 alone inhibits bacterial growth.

Results of this research and other reports (5, 9, 14, 19, 20) indicate CO_2 has potential as an inhibitor of bacterial growth in refrigerated milk. Treatment with CO_2 decreases growth rates of psychrotrophs and other groups of bacteria. Storage-life of refrigerated milk can be extended 1 to 3 days or longer depending upon initial microbiological quality. In addition, concentrations of CO_2 below 30 mM do not to diminish the processing quality of milk (9). Furthermore, CO_2 can be easily removed from milk prior to processing by vacreation, or gentle agitation (9, 14). Also, CO_2 is a normal component of freshly drawn milk (9)

making it more acceptable to regulatory agenceys and consumer groups than other less "natural" preservatives.

ACKNOWLEDGMENTS

Appreciation is extended to W. L. Tucker for assistance with statistical analyses.

This research was supported by a funds from a Grant-In-Aid of Research from Sigma Xi The Scientific Research Society, Newhaven, CT and from the South Dakota State University Graduate Research Fund.

REFERENCES

1 Atherton, H. V., and J. A. Newlander. 1977. Chemistry and testing of dairy products. 4th ed. AVI Publ. Co., Westport, CT.

2 Anonymous. 1977. Difco Manual of dehydrated culture media. 9th ed. Difco Laboratories., Detroit, MI.

3 Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products. A Review. J. Food Prot. 45:172.

4 Daniels, D. M., R. Krishnamurthi, and S. S. H. Rizvi. 1985. A review of the effects of carbon dioxide on microbial growth and food quality. J. Food Prot. 48:532.

5 Duthie, C. M., W. F. Shipe, and J. H. Hotchkiss. 1985. Effect of low level carbonation on the keeping quality of processed milk. J. Dairy Sci. 68(Suppl. 1):69 (Abstr.).

6 Fairbairn, D. J., and B. A. Law. 1986. Proteinases of psychrotrophic bacteria: Their production, properties, effects, and control. J. Dairy Res. 53:139.

7 Gill, C. O., and K. H. Tan. 1979. Effect of carbon dioxide on growth of Pseudomonas fluorescens. Appl. Environ. Microbiol. 38:237.

8 Gerhardt, P. (Ed.). 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC.

9 King, J. S., and L. A. Mabbitt. 1982. Preservation of raw milk by the addition of carbon dioxide. J. Dairy Res. 49:439.

10 King, A. D., and C. W. Nagel. 1967. Growth inhibition of Pseudomonas by carbon dioxide. J. Food Sci. 32:575.

11 King, A. D., and C. W. Nagel. 1975. Influence of carbon dioxide upon the metabolism of Pseudomonas aeruginosa. J. Food Sci. 40:362.

12 Krieg, N. R., and J. G. Holt. 1984. Bergey's manual of systematic bacteriology. Vol. 1. Williams and Wilkins, Baltimore, MD.

13 Law, B. A. 1979. Reviews of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46:573.

14 Mabbitt, L. A. 1982. Preservation of refrigerated milk. Kiel. Milchwirtsch. Forschungsber. 34:28.

15 Marth, E. H. (Ed.). Standard methods for the examination of dairy products. 14th ed. Amer. Publ. Health Assoc., Washington, DC.

16 Martley, F. G., S. R. Jayashanker, and R. C. Lawrence. 1970. An improved agar medium for the detection of proteolytic organisms in total bacterial counts. J. Appl. Bacteriol. 33:363.

17 Repaske, R., and M. A. Clayton. 1978. Control of Escherichia coli growth by CO_2. J. Bacteriol. 135:1162.

18 Sears, D. F., and R. M. Eisenberg. 1961. A model representing the physiological role of CO_2 at the cell membrane. J. Gen. Physiol. 44:869.

19 Shipe, W. F., G. F. Senyk, E. J. Adler, and R. A. Ledford. 1982. Effect of infusion of carbon dioxide on the bacterial growth in fluid milk. J. Dairy Sci. 65(Suppl. 1):77 (Abstr.).

20 Skudra, L. A. 1983. Effect of carbon dioxide on the microflora of milk. Trudy, Latviiskaya Ordena Trudovogo Krasnogo Znameni Sel'skohozyaistvennaaya Akademiya (1982) No. 196, 3-15.(Dairy Sci. Abstr. 45:268).

21 Steel, R. G. D., and J. H. Torrie. 1960. Principals and procedures of statistics. 2nd. ed. McGraw-Hill Book Co., Inc. New York, NY.

22 Thomas, S. B., R. G. Druce, and M. Jones. 1966. The significance of psychrotrophic bacteria in raw milk. Dairy Ind. 31:27.

23 Valley, G., and L. F. Rettger. 1927. The influence of carbon dioxide on bacteria. J. Bacteriol. 14:101.

TABLE 1. Characterization of psychrotrophic bacteria isolated from raw milk samples.

	Bacterial isolate				
	1p	2p	11p	12p	17p
CHARACTERISTIC					
Gram reaction	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Motility	P[1]	P	P	P	P
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Fluoresent pigment	+	+	-	-	-
Growth at 5°C	+	+	+	+	+
Growth at 41°C	-	(+)[2]	-	-	-
O/F glucose	O[3]	O	O	O	O
Xylose	+	+	+	+	+
Urea hydrolysis	+	-	+	-	-
Citrate utilization	+	+	+	+	+
Indole	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+
Nitrate reduction	-	G[4]	-	-	+
Casein hydrolysis	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	-
Lipolytic activity	+	+	+	+	-
IDENTIFICATION	--Pseudomonas fluorescens--		----Pseudomonas species----		

1 P = Motile by polar flagella.
2 (+) = Indicates a weak positive reaction.
3 O = Indicates aerobic utilization of glucose.
4 G = NO_3 reduced to N_2.

TABLE 2. Carbon dioxide concentrations[1] used in growth experiments in whole milk inoculated with pure cultures and incubated at 7°C.

Bacterial isolate	Initial	Final
	mM	
1p	21.5[2] ± 1.9	21.3[2] ± 1.6
2p	21.6 ± 1.8	21.3 ± 1.2
11p	25.4 ± 2.3	24.2 ± 1.0
12p	24.7 ± 2.1	23.9 ± 1.4
17p	24.1 ± 1.5	24.0 ± 2.7

[1] Mean ± standard deviation.
[2] N = 18.

TABLE 3. Initial and final pH values[1] for milks used in growth experiments with pure cultures incubated at 7°C.

Bacterial isolate	Treatment	Initial	Final	Net change
		pH		
1p	Air	6.60[2]	6.58[3]	-.02
	CO_2		6.10[4]	-.50
2p	Air	6.59	6.63	.04
	CO_2		6.20	-.39
11p	Air	6.70	6.26	-.44
	CO_2		6.22	-.48
12p	Air	6.62	6.51	-.11
	CO_2		6.22	-.40
17p	Air	6.65	6.60	-.05
	CO_2		6.28	-.37

[1] Mean values.
[2] N = 3.
[3] N = 6.
[4] N = 9.

TABLE 4. Generation times (GT) and percent uninhibited growth rate for isolates incubated at 7°C in whole milk and whole milk treated with CO_2[1].

Bacterial isolate	Generation Time		% Uninhibited growth rate[2]
	Air	CO2	
	-------h-------		-------%-------
1p	6.3	16.7**	37.5
2p	6.7	14.3**	46.7
11p	5.3	14.3**	36.8
12p	7.1	14.3**	50.0
17p	7.7	14.3**	53.8

[1] Concentrations of CO_2 are given in Table 2.

[2] GT Air / GT CO_2 X 100.

** Significantly different from GT in Air ($P < .01$).

TABLE 5. Carbon dioxide concentrations[1] used in growth experiments with naturally contaminated raw milk incubated at 7°C.

Farm number	Initial	Final
	----------mM----------	
1	26.5[2] ± .5	28.4[2] ± .7
2	27.3 ± 1.4	28.5 ± 1.8
3	25.3 ± .6	25.0 ± 1.1
4	28.2 ± 1.9	25.5 ± 1.0

[1] Mean ± standard deviation.
[2] N=4

TABLE 6. Comparison of microbial populations in raw milks with and without added CO_2 incubated 144 h at 7°C.

Farm number	Treatment	PC-25[1]	Coliforms	PBC[2]	Facultative and obligate anaerobes
		CFU/ml			
1	Air	620,000,000	1,100,000	640,000,000	290,000,000
	CO_2	11,000,000**	40,000**	12,000,000**	490,000**
2	Air	220,000	< 100	280,000	1,300
	CO_2	1,500**	< 100	350**	800**
3	Air	360,000,000	7,000	470,000,000	46,000
	CO_2	240,000**	200**	260,000**	2,300*
4	Air	290,000,000	2,200	330,000,000	3,200,000
	CO_2	1,700,000**	180**	1,800,000**	1,300,000**

[1] PC-25 = Plate count incubated at 25°C.
[2] PBC = Psychrotrophic bacterial count.
** Different from air control $P<.01$.
* Different from air control $P<.05$.

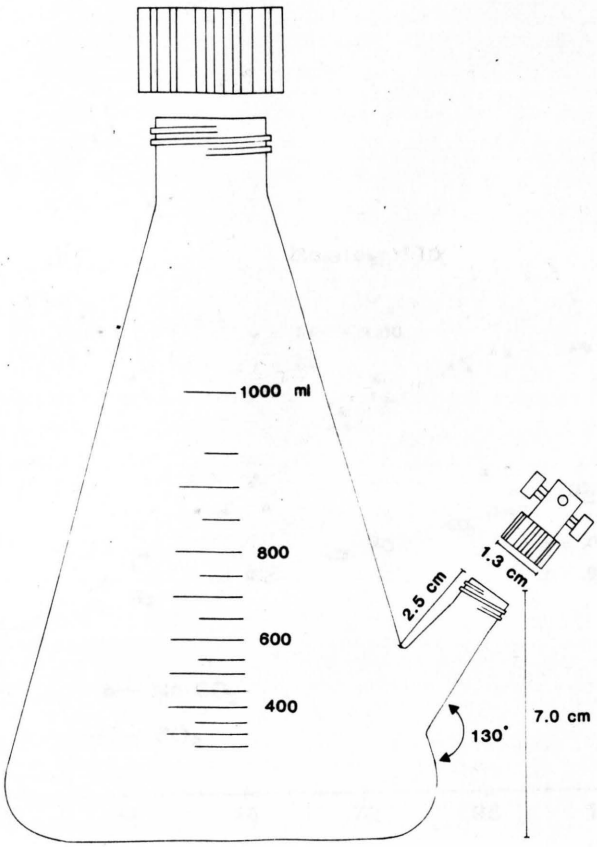


Figure 1. Diagram of the growth flask used to evaluate bacterial growth in refrigerated milk held under atmospheres containing CO_2.

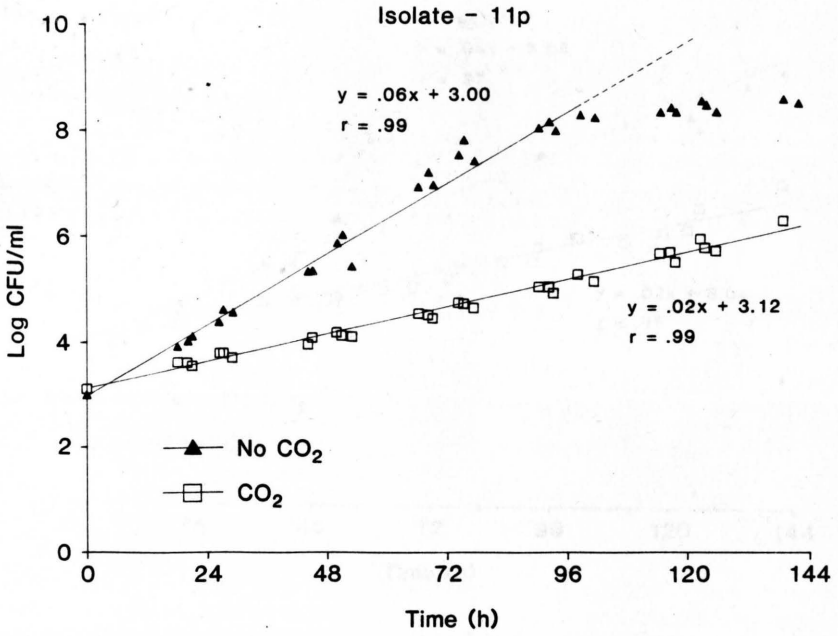


Figure 2. Growth of isolate 11p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.

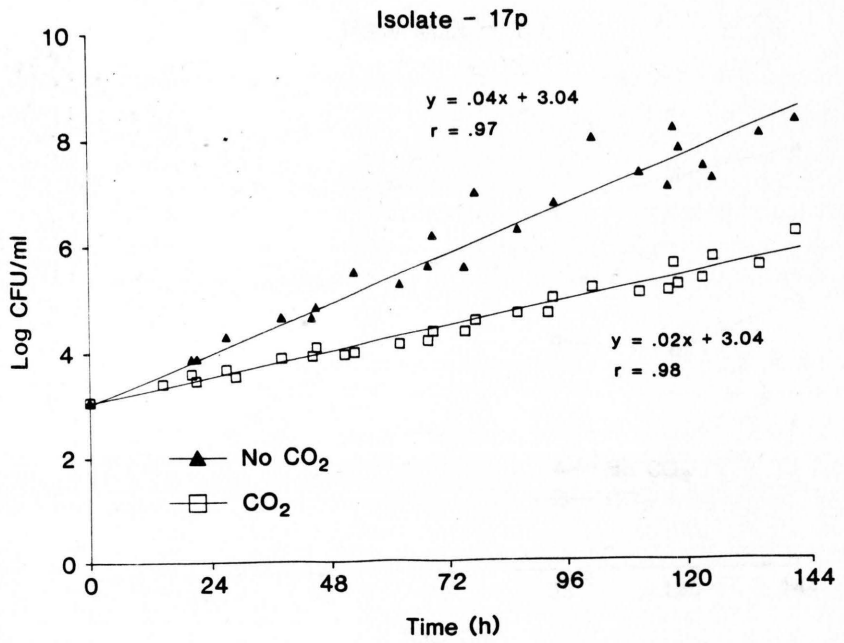


Figure 3. Growth of isolate 17p incubated at 7°C in milk and milk treated with CO_2. Equations represent pooled data from 3 separate experiments.

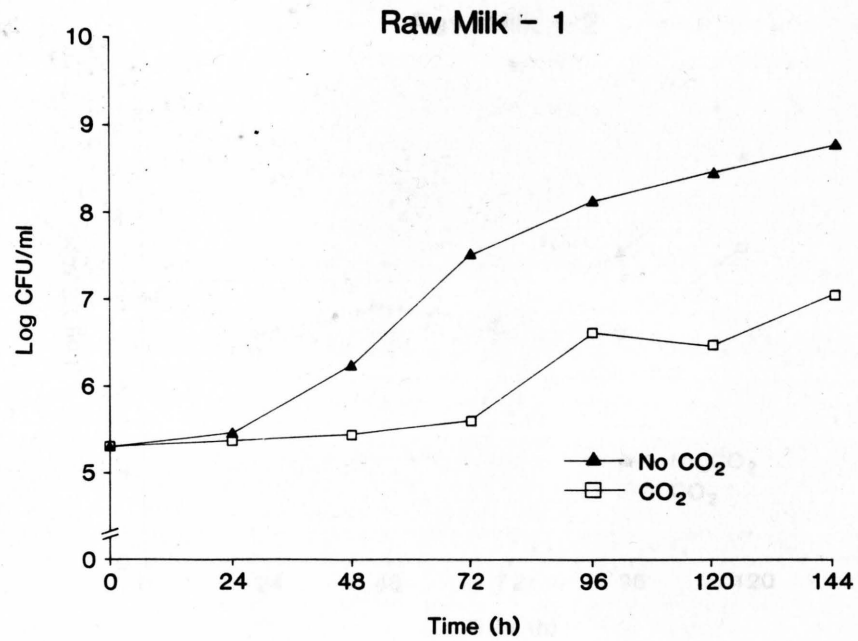


Figure 4. Aerobic plate counts in Raw Milk-1 with and without added CO_2 incubated for 144 h at 7°C.

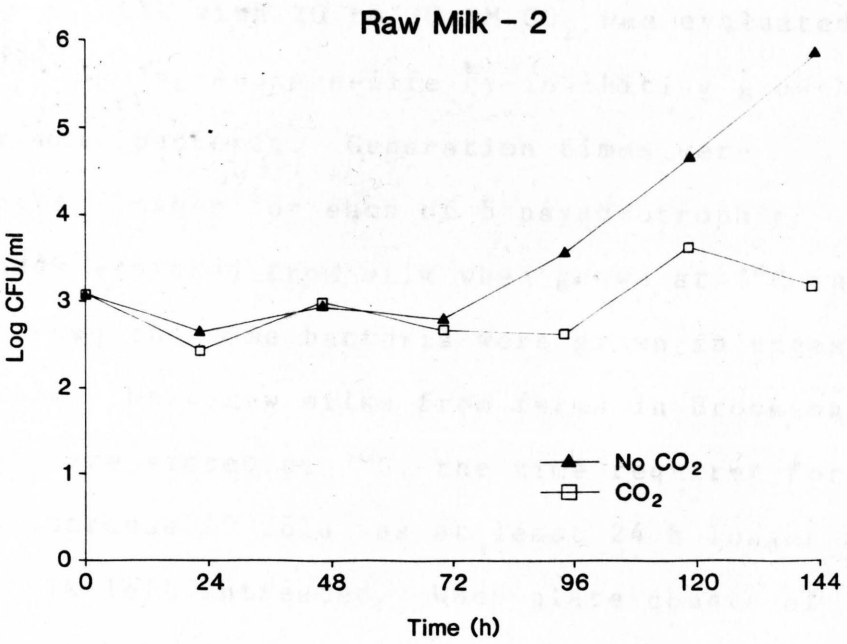


Figure 5. Aerobic plate counts in Raw Milk-2 with and without added CO_2 incubated for 144 h at 7°C.

SUMMARY AND CONCLUSIONS

A method was developed for studying bacterial growth in milk treated with CO_2. Using this method, treatment of refrigerated milk with 20 to 30 mM CO_2 was evaluated as a means of extending storage-life by inhibiting growth of psychrotrophic bacteria. Generation times were significantly longer for each of 5 psychrotrophic pseudomonads isolated from milk when grown at 7°C in sterile milk than when the same bacteria were grown in ungassed sterile milk. When raw milks from farms in Brookings County, SD were stored at 7°C, the time required for plate counts to increase 10 fold was at least 24 h longer than in the same milk left untreated. When plate counts of 10^6 CFU/ml were taken as the limit of microbiological acceptabilty treatment of milk with CO_2 resulted in storage life increases of 1 to 4 days or longer depending on the initial microbiological quality of the milk. Numbers of coliforms, psychrotrophs, and anaerobes (facultative and obligate) were significantly lower in raw milks treated with CO_2 than in untreated milks after 6 days incubation at 7°C.

Organoleptic changes in milk become apparent when populations of psychrotrophic bacteria reach 10^6 CFU/ml. The ability to store refrigerated raw milk 1 to 4 days longer prior to reaching 10^6 CFU/ml would be of benefit to

the dairy industry. Smaller farms might hold milk from 3 or even 4 days milkings, provided adequate storage, without loss of quality resulting from excessive microbial growth. Manufacturing plants could hold milk recieved Friday evening until Monday morning without excessive psychrotrophic bacterial growth which could lead to yield losses. Milk produced under the every-other-day pickup system would also benefit from the preservative action of CO_2, by maintaining initial microbiological quality for longer periods.

Several questions need to be addressed before commercial application can be recommended. The influence of CO_2 on pathogens present in raw milk must be studied. For example, species of Campylobacter are known to survive longer on chicken packaged under CO_2. Other pathogens of interest include Salmonella, Listeria, and enteropathogenic Esherichia coli. In addition to studying pathogenic bacteria, information regarding the influence of CO_2 on Gram-positive organisms in milk, particularly spore-formers, would be of interest. Studies with anaerobic bacteria such as Clostridium would also be an important contribution.

Information regarding the influence of CO_2 on processing characteristics is sparse. Studies on changes in thermal processing characteristics of milk brought about by CO_2 would be valuable. Also, the influence of CO_2 treatment on growth of lactic acid bacteria should be conducted to determine if changes occur in milk fermentation. Research

into use of CO_2 treatment prior to fluid processing, particularly organoleptic data, is essential. Other areas of potential research include: developing systems for CO_2 treatment of milk at the farm and dairy processing plant, development of methods to remove CO_2 from milk, studies on the mode of action of CO_2 as a bacteriostatic agent, and studies on the economics of CO_2 treatment of milk. Finally, research into the influence of CO_2 on current milk quality tests such as: Titratable Acidity (TA), Acid Degree Value (ADV), Standard Plate Count (SPC), and antibiotic tests must be conducted before CO_2 treatment can be recommended for use in the dairy industry.

APPENDIX A

SUPPLEMENTAL INFORMATION

TABLE 1A. Colony forming units present in Tryptic Soy Broth incubated for 18-24 h at 25°C, when absorbance at 420 nm was .3.

Bacterial isolate	CFU/ml
1p	1.54×10^8
2p	6.42×10^7
11p	7.12×10^7
12p	1.08×10^8
17p	9.61×10^7

Figure 1A. Typical chromatogram for CO_2 analyses of milk from growth flasks.

Column: 2.4 m by 1.65 mm ID Porapak Q® 100-120 mesh

Carrier Gas: He at 25 cc/min

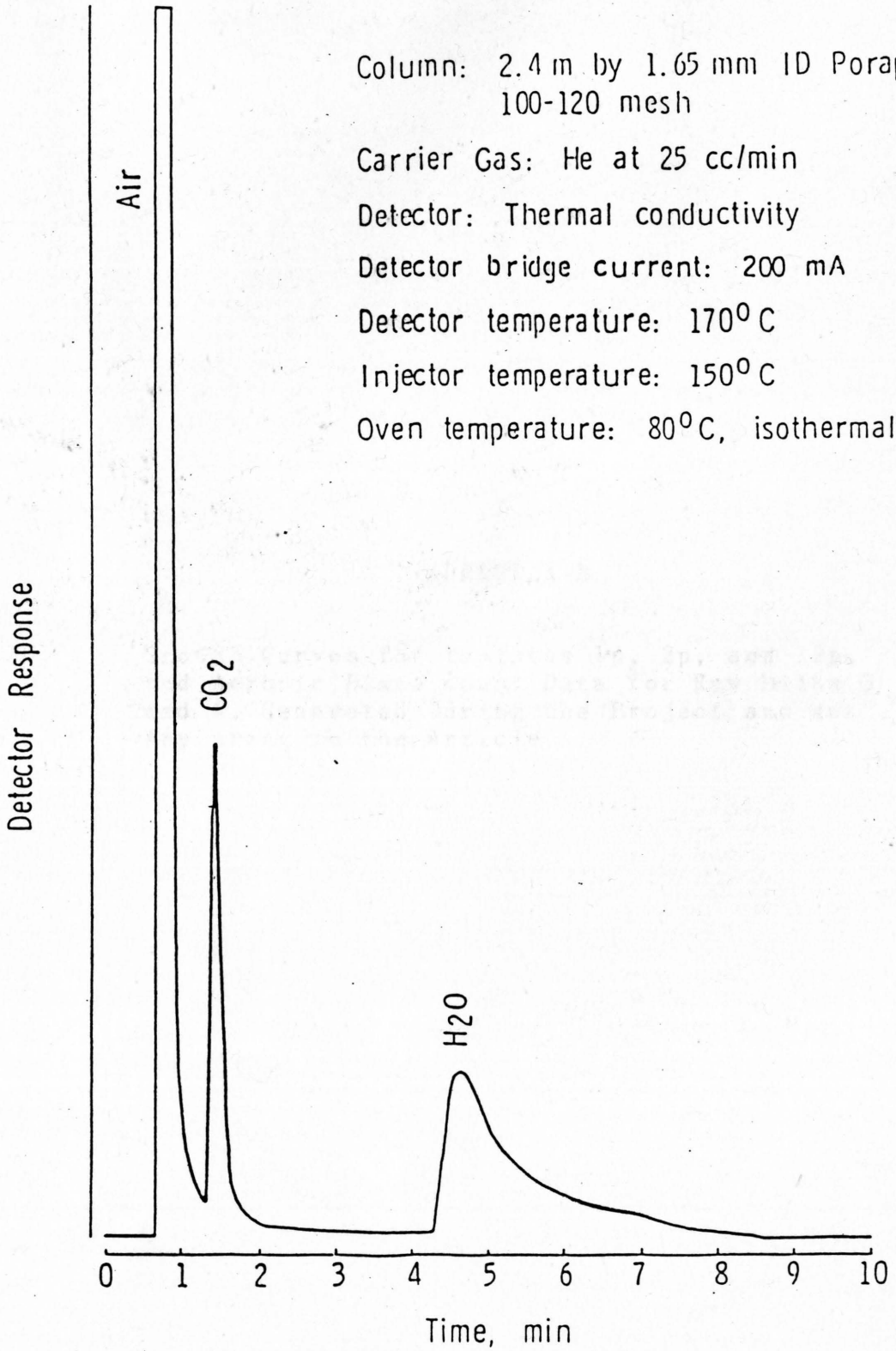
Detector: Thermal conductivity

Detector bridge current: 200 mA

Detector temperature: 170° C

Injector temperature: 150° C

Oven temperature: 80° C, isothermal



APPENDIX B

Growth Curves for Isolates 1p, 2p, and 12p, and Aerobic Plate Count Data for Raw Milks 3 and 4, Generated During the Project and not Appearing in the Article

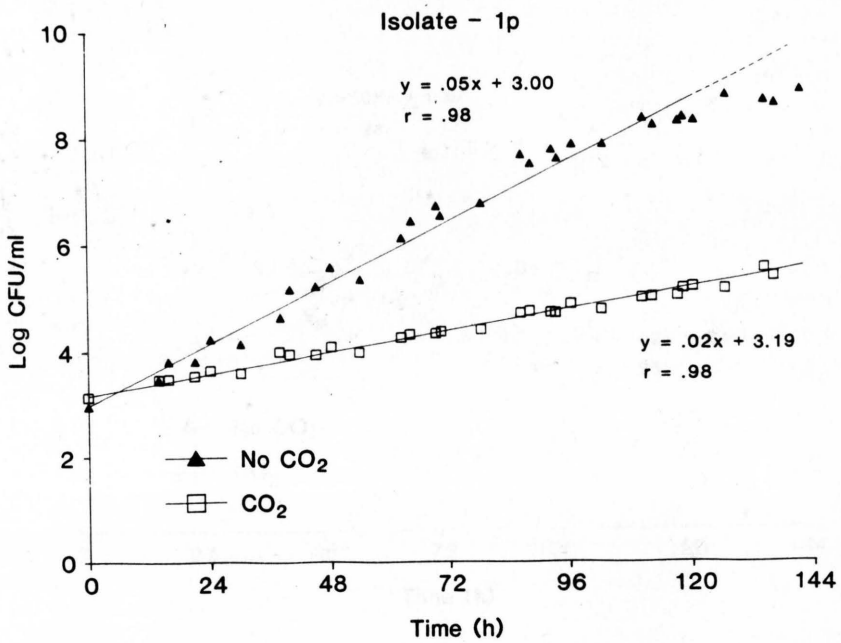


Figure 1B. Growth of isolate 1p incubated at 7°C in milk and milk treated with 21.5 mM CO_2. Equations represent pooled data from 3 separate experiments.

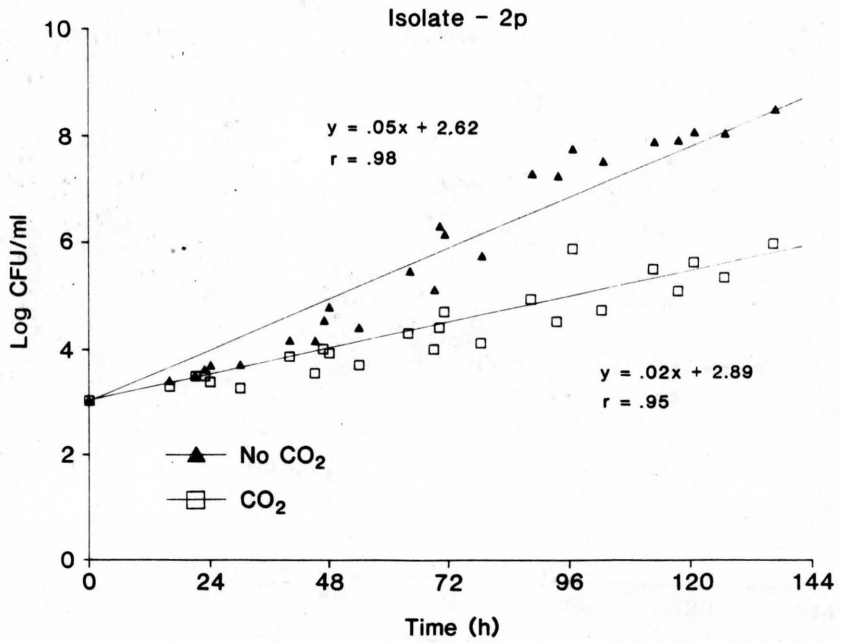


Figure 2B. Growth of isolate 2p incubated at 7°C in milk and milk treated with 21.5 mM CO_2. Equations represent pooled data from 3 separate experiments.

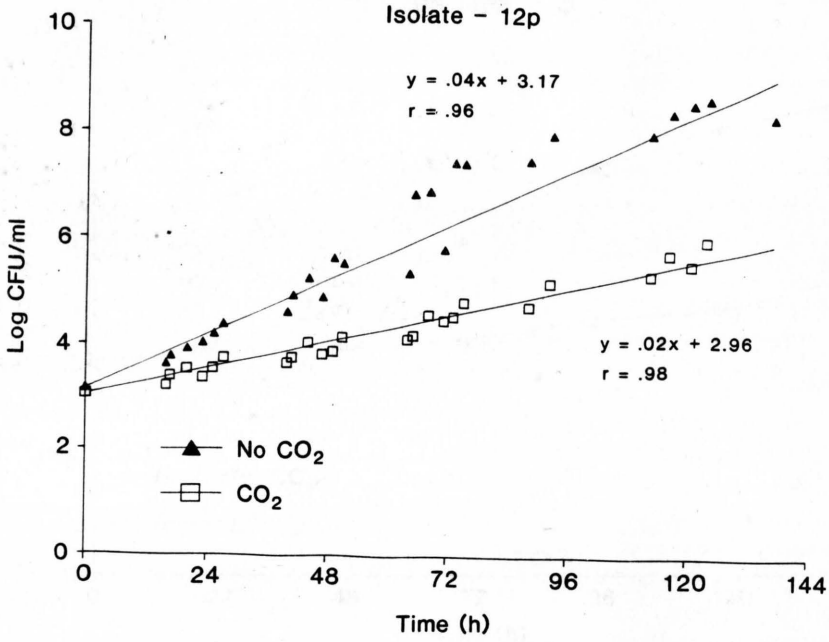


Figure 3B. Growth of isolate 12p incubated at 7°C in milk and milk treated with 24.8 mM CO_2. Equations represent pooled data from 3 separate experiments.

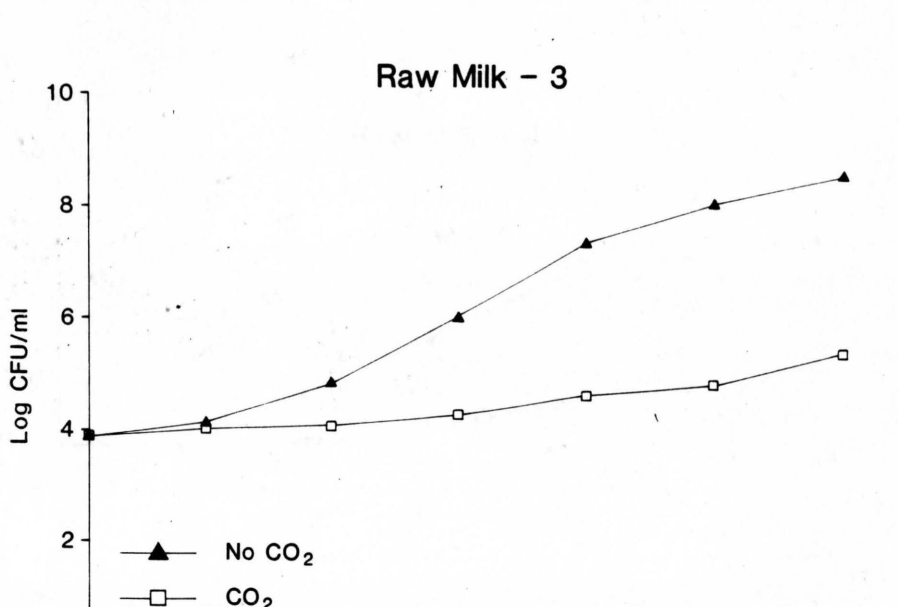


Figure 4B. Aerobic plate counts in Raw Milk-3 with and without added CO_2 incubated for 144 h at $7^{o}C$.

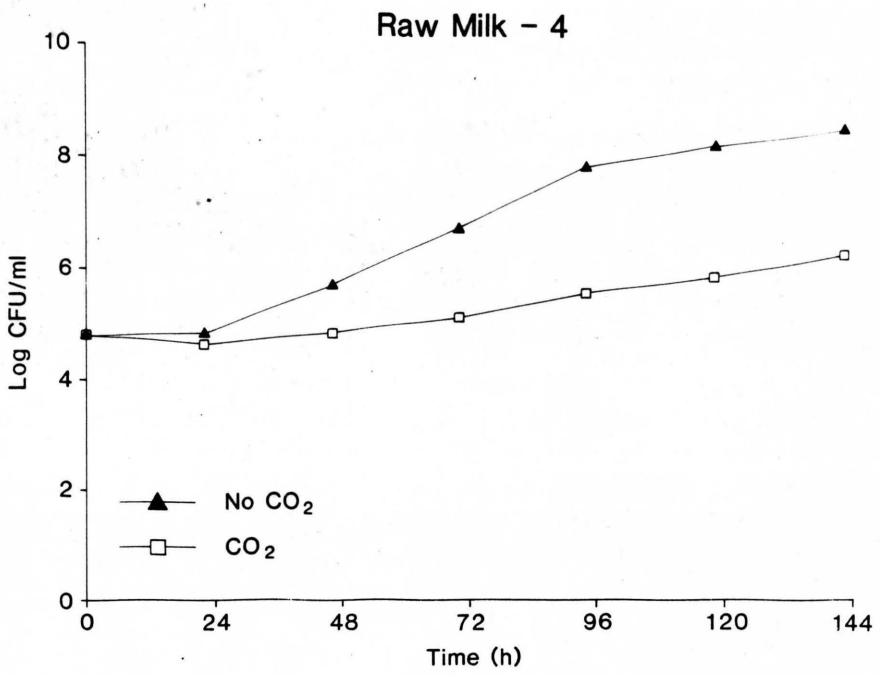


Figure 5B. Aerobic plate counts in Raw Milk-4 with and without added CO_2 incubated for 144 h at 7°C.